QUANTIFICATION OF MONOMER ELUTION FROM FOUR COMPOSITE RESINS AND ITS CYTOTOXIC EFFECT ON HUMAN GINGIVAL FIBROBLASTS – AN IN VITRO STUDY

Dissertation Submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In Partial Fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH IV

CONSERVATIVE DENTISTRY AND ENDODONTICS

APRIL 2013

CERTIFICATE

This is to certify that this dissertation titled **"QUANTIFICATION OF MONOMER ELUTION FROM FOUR COMPOSITE RESINS AND ITS CYTOTOXIC EFFECT ON HUMAN GINGIVAL FIBROBLASTS – AN IN VITRO STUDY"** is a bonafide record of work done by **DR. M. PRAVEEN KUMAR** under our guidance during the study period 2010 – 2013.

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY,** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY – CONSERVATIVE DENTISTRY AND ENDODONTICS, BRANCH IV.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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ACKNOWLEDGEMENT

I take this opportunity to express my heartfelt gratitude to my post graduate teacher, mentor and guide ***Dr. R. Indira, M.D.S., Professor and H.O.D*** *Department of Conservative Dentistry & Endodontics, Ragas Dental College, for her untiring perseverance and immense patience in motivating and supporting me throughout my postgraduate curriculum. I thank her for her guidance without which this dissertation would not have come true.*

I sincerely thank ***Dr. S. Ramachandran M.D.S.,*** *Professor and Principal, Department of Conservative Dentistry & Endodontics, Ragas Dental College, who immensely supported me during my entire postgraduate curriculum.*

I earnestly thank ***Dr. Anil Kumar M.D.S., Professor, Dr. C.S. Karumaran M.D.S., Professor, Dr. Revathi Miglani M.D.S., Professor, Dr. M. Rajasekaran M.D.S., Professor,*** *Department of Conservative Dentistry & Endodontics, Ragas Dental College and* ***Dr. Shankar M.D.S.,*** *for their guidance and valuable advice whenever I was in need.*

I would like to solemnly thank ***Dr. Veni Ashok, M.D.S., Reader*** *and* ***Dr. A.D. Senthil Kumar M.D.S*** *for all the help during my study period.*

I would like to thank ***Dr. S. M. Venkatesan, M.D.S., Dr. Shankar Narayan, M.D.S.,*** *and* ***Dr. B. Janani, M.D.S****., Senior lecturers for their friendly guidance and support.*

I wish to thank the Management of Ragas Dental College and Hospital for their help and support.

I take this opportunity to sincerely thank ***Mr K. Mohan,*** *principal scientist, Indian Institute of Chromatography and Mass Spectrometry, Chennai for helping with my HPLC analysis. He was extremely helpful, patient and interested throughout the course of the study.*

I am grateful to ***Mr A. Ganesh Kumar,*** *Chennai Dental Research Foundation for guiding me with my cytotoxicity analysis – gingival fibroblast culture and MTT assay.*

I sincerely thank ***Dr. Ravanan, Ph.D.,*** *for his guidance with the statistical analysis of this study.*

I will forever remain grateful to my batch mates who always inspired me, made me feel at home and made the three years of post-graduation a memorable and unforgettable journey. I like to extend my thanks to my juniors for their love and support.

*I would like to specially thank my **Parents** and **my brother who are the special gifts to my life** and my sister in law for their love, understanding, support and encouragement throughout these years without which, I would have never reached so far.*

*Above all, I am grateful to the **"Almighty"**, who has blessed me with such wonderful people and has given me the opportunity to seek knowledge and guiding me in my right path.*

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ABSTRACT

Background:

Monomers eluting from the composites have cytotoxic effects.

AIM:

The aim of this present study was to evaluate the amount of release of four monomers – BisGMA, UDMA, TEGDMA and HEMA from four different composite restorative materials after 24 hours, using high performance liquid chromatography and to assess the cytotoxicity of these monomers on human gingival fibroblasts by MTT assay.

METHODOLOGY:

The four composites analysed in this study were microhybrid (Filtek Z100), Ormocer (Admira), Nanohybrid (Filtek Z250 XT) and Nanocomposites (Filtek Z 350 XT). Eight samples from each composites were made in Teflon moulds of 5×2 mm and cured with halogen light for 40 s. All samples were immersed in 2 ml of 75% ethanol and incubated at 37°C for 24 hours. At the end of 24 hours the samples were removed, solution analysed by HPLC and the mean concentrations of monomers were calculated. The cytotoxicity of these monomers were assessed on human gingival fibroblast by MTT assay.

RESULT:

High quantity of BisGMA was eluted from all the composites followed by UDMA except in microhybrid composite. HEMA was eluted in minimum quantity from all the four composites. Only microhybrid composite eluted higher amounts of TEGDMA. When the cytotoxicity of these monomers were assessed, BisGMA was the most cytotoxic monomer compared to the other monomers due to its high amount of release followed by UDMA and TEGDMA. HEMA was the least cytotoxic.

CONCLUSION:

Nanocomposite Filtek Z350 XT (3M ESPE) eluted the maximum amount of monomers at the end of 24 hours compared to the other three composites. BisGMA was the most cytotoxic monomer compared to other monomers due to its high amount of release.

Keywords: Composites, monomers, high performance liquid chromatography (HPLC), cytotoxicity, human gingival fibroblast, MTT assay.

Introduction

INTRODUCTION

In contemporary dental practice, the concept of esthetics has been given prime importance. There is a revolution in dentistry where evolution of newer esthetic dental materials has conquered the place of dental amalgam.

The development of composites as a restorative material is a big boon to the restorative field as it had the answer to the increasing demand of esthetic restorative material for the past few years. Composites are perfect alternatives for amalgam and ceramic restorations. They have a wide variety of clinical applications in the field of dentistry as a direct filling material, inlays and onlays, bonding agents, crowns and bridges, temporary crowns and endodontic filling.[13]

According to Dental Clinics of North America, composites are defined as three dimensional combination of atleast two chemically different materials with two distinct interfaces separating the components. The dental composites are primarily composed of two parts namely the organic part and the inorganic part. The organic part includes the polymerizable resin matrix and the inorganic part

includes the filler particles. This organic polymerizable resin matrix and inorganic filler particles are bonded together by a silane coupling agent. In addition to these components the composite material also contains photoinitiators, co-initiators, inhibitors of polymerisation and photostabilisers.[17]

The most commonly used polymerisable resin matrix in the composites are the methacrylates such as BISGMA, UDMA, TEGDMA and HEMA. But recently a new resin system such as Ormocers have been introduced.[46] The fillers include silica, glass, quartz or ceramic material. The physical and chemical properties of the composite material is mainly influenced by the filler content, filler size and distribution of filler particles.[34]

The resin content of the composites which is composed of the monomers are converted into highly cross – linked polymers on exposure to light sources that generate the formation of free radicals thus propagating the polymerisation reaction resulting in a set material[4]. The various light sources available in the market today are the Halogen, LED, Plasma arc curing units and the Laser. These light curing units differ in their wavelength and intensity of curing.[29]

The conversion of monomer to polymer during polymerisation which is termed as the degree of conversion is always not complete. Literatures give evidence that there is only about 40% - 75% conversion of monomer to polymer occur during polymerisation. The remaining monomers are trapped within the polymers as the unreacted monomers.[22] Various factors which influence the degree of conversion are composition of monomer, concentration of activator and inhibitor present, viscosity of monomers, diffusion limitation of reactive media present, size and shape of filler particles, light intensity of the curing unit, duration of light irradiation, temperature produced during polymerisation and thickness of restorative material used[11]

These unreacted monomers elute from resin based composites as a result of chemical biodegradation in the presence of liquids such as water, saliva, ethanol, methanol, acetonitrile and bacterial enzymes.[10,17,22] The elution of unreacted monomer in addition to compromising the physical and mechanical properties of the material also act as plasticizers, decreasing the mechanical strength, dimensional stability, clinical serviceability and allow bacterial growth due to the ingress of oral fluids.[7,41]

The unreacted monomers also questions the biocompatibility of composite material. The allergenic properties of the monomers are well exposed by earlier studies.[24] Further the monomers also cause cytotoxicity,[1,19,36] genotoxicity,[9] mutagenicity[40] and toxic reactions to the reproductive system.[24,26] It causes major cytotoxic reactions to the dental pulp and gingival fibroblasts.[18,25,27] The cytotoxicity can be assessed using various assays. MTT assay is the most commonly used assay to check the cell viability which converts water soluble methylthiazole tetrazolium bromide to an insoluble purple formazan.[18,28]

AIM:

The aim of this present study was to evaluate the amount of release of four monomers – BisGMA, UDMA, TEGDMA and HEMA from four different composite restorative materials at the end of 24 hours, using high performance liquid chromatography and to assess the cytotoxicity of these monomers on human gingival fibroblasts by MTT assay.

OBJECTIVES:

1. To quantify the amount of monomers eluted from four different resin composites at the end of 24 hours.
2. To prove that different composite resins elute different quantity of monomer.
3. To evaluate the cytotoxic effect of these monomers on Human Gingival Fibroblasts by MTT assay.

Review of literature

REVIEW OF LITERATURE

Ferracane et al (1990)[10] studied the uptake of solvent and the elution of molecules from a dental composite and an unfilled resin which were monitored with time during soaking in either water or an ethanol/water mixture. The results showed that approximately 50% of the leachable species were eluted from the composite within three hours of soaking in water, while 75% of the leachable molecules were eluted into the ethanol/water mixture. Elution of nearly all of the leachable components was complete within a 24-hour period in either solvent. The study lends support to the view that dental composites do not provide a chronic source of unreacted monomer to the pulp or other oral tissues, due to a rapid and complete elution of the molecules.

Ratanasathien et al (1995)[36] investigated the effects of four dentin bonding components – HEMA, TEGDMA , UDMA , BisGMA and their interactive combinations on Balb/ c 3T3 mouse fibroblasts using MTT assay for 24 hour and 72 hour exposure. The monomers are ranked on the basis of cytotoxicity as BisGMA > UDMA > TEGDMA > HEMA. The cytotoxicity of the components increased with longer period of exposure. It is concluded that both period of

exposure and interaction between the components play an important role in determining the cytotoxicity.

Spahl et al (1998)[44] determined the quality and quantity of leachable residual (co) monomers and additives eluted from various commercial dental composite resins after polymerization. In all polymerized composite resin specimens, (co) monomers and various additives as well as contaminants from manufacturing processes were identified. Almost every compound detected in the unpolymerized resins could also be identified in the methanol extracts, but only a few of them were found in the water extracts. From these the co-monomer TEGDMA was extracted in quantities higher than those reported to be cytotoxic in primary human oral fibroblast cultures. It was concluded that the extractable quantities of composite resin components should be minimized, either by reducing the mobility of leachable substances within the set material or by applying less water-soluble components.

Schuster et al (1999)[39] hypothesized that HEMA is cleaved and release ethylene glycol which is incorporated into cell lipids, yielding phosphatidylethylene glycol (PtEG) and the methacrylic acid alters other lipid pathways in a manner similar to that of methacrylic

acid released from hydrolysis of DMAEMA. In the presence of HEMA several classes of lipids were altered. Among the neutral lipids, the most notable changes involved sterol precursors, triglycerides, fatty acids, and cholesterol esters, while phosphatidylcholine was affected among the phospholipids. The results differed quantitatively between the two cell types. Results also suggest that EG, including that released by hydrolysis of HEMA, is incorporated into cell phospholipids, producing PtEG. The changes in neutral lipid labelling may occur by alteration of lipid synthetic pathways utilizing acetyl Co-A as well as inhibition of enzymes involved in synthesis of cholesterol from sterol precursors and hydrolysis of cholesterol esters. Synthesis of PtEG may take place via phospholipase D-mediated head group exchange. Alterations in the cellular lipids may affect cell membrane properties and associated cell functions.

Munksgaard et al (2000)[30] compared the elution of monomers BisGMA and TEGDMA from a commercial resin composite (Z-100) and an experimental resin when cured with halogen light and plasma arc unit by using High Performance Liquid Chromatography. The elution of monomers from experimental resin and resin composite

was 7 and 4 times higher when cured with plasma arc unit compared to halogen light. It was concluded that plasma arc unit doesn't provide the required curing as recommended by the manufacturer.

Ortengren et al (2001)[32] assessed the water sorption , solubility and monomer elution from six different composite materials at various time intervals of 4 h, 24 h, 7 days, 60 days and 180 days. Water sorption increased for all composite materials until equilibrium. The water solubility behaviour varied for each composite material. HPLC analysis revealed that the TEGDMA was the main monomer eluted, with quantifiable quantities of UDMA and detectable amounts of BisGMA. Maximum monomer elution was observed after 7 days.

Sideridou et al (2001)[41] studied the room-temperature photopolymerization of Bis-GMA, Bis-EMA, urethane dimethacrylate (UDMA) and triethylene glycol dimethacrylate (TEGDMA) induced by camphoroquinone/N;N dimethylaminoethyl methacrylate, as photoinitiator system, was followed by FT-IR. The latter was found to increase in the order Bis-GMA<Bis-EMA<UDMA<TEGDMA. The photopolymerization of mixtures of Bis-GMA/TEGDMA, Bis-GMA/UDMA and Bis-GMA/Bis-EMA

showed a good linear relationship of degree of conversion with the mole fraction of Bis-GMA and in the case of the first pair also with the Tg of the initial monomer mixture.

Santerre et al (2001)[37] reviewed the principal modes of dental composite material degradation and related them to the specific components of the composites like monomer resins, the filler content, and the degree of monomer conversion after the clinical materials are cured. Loss of mechanical function, leaching of components from the composites and the impact of biodegradation on the ultimate biocompatibility of current materials is discussed.

Kehe et al (2001)[19] investigated the cytotoxic potentials of the dental composite components triethyleneglycoldimethacrylate (TEGDMA) and 2-hydroxy-ethylmethacrylate (HEMA) as well as mercuric chloride ($HgCl_2$) and methyl mercury chloride (MeHgCl). Proliferating A549 and L2 cell monolayers were cultured in the absence or presence of composite components or mercurials. The EC50 values of both mercurials were significantly ($P<0.05$) lower compared to the values of both composite components. TEGDMA was about 5-fold (A549 cells) and about 2-fold (L2 cells) more toxic

compared to HEMA. It is to be assumed that the risk of lung cell damage by dental composite components is even more unlikely.

Stansbury et al (2001)[45] determined the validity and practicality of near infrared (NIR) spectroscopic techniques for measurement of conversion in dental resins. The conversion of 3 mm thick photopolymerized Bis-GMA/TEGDMA resin specimens was determined by transmission NIR. Specimens were then ground and reanalyzed in KBr pellet form by mid-IR. Conversion values obtained by NIR and mid-IR techniques did not differ significantly. The non destructive analysis of conversion in dental resins by NIR offers advantages of convenience, practical specimen dimensions and precision compared with standard mid-IR analytical procedures.

Deb Sanjukta et al (2003)[6] compared the effect of plasma light curing using 3 s and step cure regime with halogen light curing on the properties four different restorative materials. It was concluded that properties obtained with 3 s plasma light curing was inferior to those obtained with step cure regime and halogen light curing.

Michelsen et al (2003)[23] identified the organic elutes from two restorative composites, one compomer and one RMGIC using gas chromatography – mass spectrometry. About thirty two substances

such as monomers, co-monomers, initiators, stabilizers and other products were identified. These different organic elutes will have various effects on the biocompatibility of the materials.

Finer et al (2004)[13] studied the biomolecular interactions between composite resin chemistry and esterase activity to explain the differences in biodegradation levels between the ubis and bis resin systems by analyzing the degradation products using high-performance liquid chromatography, UV spectroscopy and mass spectrometry. Both materials were characterized by Fourier transform infrared spectroscopy, scanning electron microscopy and X-ray photoelectron spectroscopy. Because both systems were identical except for their monomer systems, it was concluded that changes in biostability were associated with chemistry.

Moon et al (2004)[29] evaluated the effect of the three curing units – halogen , plasma arc , LED with different irradiation protocols (one-step , two-step and pulse) on the elution of BisGMA , UDMA and surface hardness of composite resins. Elution of monomers was assessed by HPLC and surface hardness by Vicker's hardness number (VHN) after immersion of samples in ethanol for 7 days. The results show that when the light energy density is less than 17 J/cm^2 there is

a difference in the VHN and amount of monomer elution exhibited by the three curing units and different irradiation protocols. But when the time and light energy density is increased the difference is less, irrespective of the curing units and irradiation protocols.

Issa et al (2004)[18] investigated the cytotoxicity of composite resin monomers on human gingival fibroblast culture by using MTT and LDH assay. The cytotoxicities shown by the monomers in MTT and LDH assay are similar. The monomers are ranked based on their TC 50 concentrations as BisGMA > TEGDMA > DMAEMA > HPMA > HEMA. It is concluded that a variety of toxic reactions are shown by the resin monomers on human gingival fibroblasts.

Lefeuvre et al (2004)[21] investigated the effects on glutathione (GSH) level and glutathione transferase P1 (GSTP1) activity in cultured human gingival fibroblasts. TEGDMA cytotoxic concentrations (from 0.5 to 2 mM) induced a depletion of GSH without formation of oxidized GSH (GSSG). In fibroblasts expressing the wild-type GSTP1, TEGDMA both inhibited and potentiated GSTP1 activity at high (IC50 = 1.1 mM) and low concentrations, respectively. In contrast, cells expressing the GSTP1 *A/*B variant showed a weak inhibition of GST activity only, associated with

greater sensitivity to drug toxicity. Biochemical analysis of GSTP1 inhibition revealed that TEGDMA is a non-competitive antagonist with respect to GSH and substrate. Thus, TEGDMA interference with GSH and GSTP1 activity may contribute to dental-resin-induced adverse effects.

Spagnuolo et al (2004)[43] examined apoptosis and necrosis induced by TEGDMA in human primary pulp cells. The levels of apoptotic and necrotic cell populations differentially increased after exposure to increasing concentrations of TEGDMA. A two-fold increase in the percentage of apoptotic cells was induced by 1 mmol/L TEGDMA. However, a population shift among cells in apoptosis and necrosis was detected when cell cultures were exposed to 2 mmol/L TEGDMA. Akt phosphorylation was inhibited in the presence of TEGDMA. The results suggest that depression of PI3K signalling may be a primary target in TEGDMA-induced apoptosis.

Komurcuoglu et al (2005)[20] determined the concentration of residual monomers and to evaluate the effectiveness of elimination methods of residual monomers in three different fissure sealant materials (Helioseal F, Filtek Flow and EXM-510). High performance liquid chromatography was used to determine the

concentrations of residual monomers. Results of the study showed that residual Bis-glycidyl dimethacrylate elution was the highest in Helioseal F and the lowest in Filtek Flow with the three methods tested. For triethleneglycol dimethacrylate, EXM-510 eluted the highest residual monomer. It was also found that although the three tested methods were insufficient for removing all of the residual monomers and rubbing with cotton rolls was more effective than other two methods.

Siderisou et al (2005)[42] studied the elution of residual monomers from light-cured dental resins and resin composites into a 75% ethanol:water solution using High-Performance Liquid Chromatography (HPLC). The resins studied were made by light-curing of Bis-GMA, TEGDMA, UDMA, Bis-EMA and mixtures of these monomers. The resin composites were made from two commercial light-cured restorative materials (Z100 MP and Filtek Z250), the resin matrix of which is based on copolymers of these monomers. The effect of the curing time on the amount of monomers eluted was investigated. The concentration of the extractable monomers was determined at several immersion periods from 3 h to 30 days. For all the materials studied, it was observed that the

chemical structure of the monomers used for the preparation of the resins, which defines the chemical and physical structure of the corresponding resin, directly affects the amount of eluted monomers, as well as the time needed for the elution of this amount. In the case of composites, it seems that the elution process is not influenced by the presence of filler.

Witzel et al (2005)[47] investigated the influence of photoactivation method on various properties such as flexural strength (FS) , degree of conversion (DC) , flexural modulus (FM) and knoop hardness (KHN) of a composite (Filtek Z250) and an unfilled resin (Scotchbond multi-purpose plus) after storage in ethanol or water. The composite properties and its susceptibility to ethanol degradation are not affected by photoactivation method. However the low intensity curing produced lower DC in unfilled resin and reduced FS after ethanol storage.

Schweikl et al (2005)[40] investigated cytotoxic effects and the formation of micronuclei in V79 fibroblasts after exposure to extracts of modern composite filling materials (Solitaire, Solitaire 2, Tetric Ceram, Dyract AP, Definite) For cytotoxicity testing, test specimens were aged for various time periods (0, 24, and 168 h), and V79 cells

were then exposed to dilutions of the original extracts for 24, 48, and 72 h. The ranking of the cytotoxic effects of the composites according to EC50 values after a 24-h exposure period was as follows: Solitaire (most toxic) = Solitaire 2 < Tetric Ceram < Dyract AP < Definite (least toxic). Cytotoxicity was independent of the period of aging for each composite, but varied with exposure periods. It was concluded that mutagenic components of biologically active composite resins should be replaced by more biocompatible substances to avoid risk factors for the health of patients and dental personnel.

Nalcaci et al (2006)[31] measured elution of monpomers TEGDMA and BisGMA from hybrid and micro-filled composites cured with two different light sources – QTH and LED for various time intervals ranging from 0 to 72 hours. High levels of TEGDMA elution was noted in samples cured with standard QTH compared to samples cured with high – intensity QTH and standard LED. Majority of TEGDMA eluted within 9 hours irrespective of the different polymerization regime. BisGMA elution showed no significant difference regardless of different curing protocols upto 72 hours.

Floyd et al (2006)[14] studied double bond conversion, polymer network formation and leachable portion from two polymeric systems

– UDMA/TEGDMA and BisGMA/TEGDMA. It was found that UDMA polymer system showed significantly higher double bond conversion and crosslinking than BisGMA polymer system. Also higher elution of unreacted monomers in BisGMA mixture than the UDMA system.

Garcia et al (2006)[15] reviewed different components of the composites currently used in dentistry. Most composites used in dentistry are hybrid materials as they are composed of polymer groups reinforced by an inorganic phase of glass fillers with different compositions, particle sizes and fill percentages. Both halogen lamps, whether conventional or high intensity, and LED curing lights which provide a gradual increase in light intensity are very useful for reducing shrinkage of the composite material. The clinical choice of a composite must consider whether priority should be given to mechanical or aesthetic requirements: if mechanical considerations are paramount the material with the greatest volume of filler will be chosen; if aesthetic considerations predominate, particle size will be the most important factor.

Polydorou et al (2007)[33] determined the elution of monomers from hybrid (Tetric Ceram) and flowable (Tetric Flow) resin

composites after different polymerization times of 0 s , 20 s , 40 s , 80 s stored for 24 hours, 7 days and 28 days. BisGMA elution was compared to TEGDMA regardless of different polymerization and storage times. Total monomer elution was significantly higher in hybrid composite than the flowable material. There is no difference in monomer elution when polymerized at 20 s and 40 s but curing with 80 s showed less monomer elution. After 28 days there is decraese in release of TEGDMA but BisGMA remained at high levels.

Darmani et al (2007)[5] investigated the components released and cytotoxicity of four different resin based composite materials (Z100, Solitaire 2, Filtek P60 and Synergy). The components release was evaluated using High Performance Liquid Chromatography. Cytotoxicity was assessed by MTT assay using Balb/c 3T3 fibroblasts. By HPLC analysis varying concentrations of BisGMA, TEGDMA, UDMA, bis-EMA and bisphenol A were obtained from the different composite materials. The composites and the eluted substances had cytotoxic effects on the fibroblasts. Among the composites, Synergy was less toxic and Solitaire 2 was more toxic.

Beun et al (2007)[2] compared the mechanical properties and inorganic fraction of nanofilled composites with microfilled and

universal composites. He also compared the degree of conversion of the materials when polymerized with halogen light and LED light sources. It was found that the mechanical properties of nanofilled composites are similar to that of universal composites. Also in comparing light sources for polymerization, halogen light showed higher degree of conversion compared to LED.

Moharamzadeh et al (2007)[27] compared the cytotoxic effects of three monomers – BisGMA , TEGDMA ,UDMA on human gingival fibroblast cell lines and HaCaT keratinocytes. Cell viability was assessed using Alamar Blue assay and presence of human interlukin - 1β (IL-1β) was determined by sandwich enzyme – linked immunosorbant assay (ELISA). All the three monomers showed toxic effects. It is concluded that resin monomers are toxic to human gingival fibroblasts and HaCaT keratinocytes but they were not able to induce the release of IL-1β on its own.

Michelsen et al (2008)[24] assessed the amounts of HEMA and TEGDMA eluted from two composites (Tetric EvoCeram and Filtek Z250) in human saliva for 24 h using combined gas chromatography – mass spectrometry (GC/MS) with tailor made internal standards. It

was found that TEGDMA eluted from Filtek Z250 onnly while HEMA eluted from both Tetric EvoCeram and Filtek Z250.

Goldberg et al (2008)[16] reviewed the in vitro and in vivo studies which identified that some components of restorative composite resins, adhesives and resin-modified glass ionomer cements are toxic. The mechanisms of cytotoxicity are related firstly to the short-term release of free monomers occurring during the monomer–polymer conversion. Secondly, long-term release of leachable substances is generated by erosion and degradation over time. In addition, ion release and proliferation of bacteria located at the interface between the restorative material and dental tissues are also implicated in the tissue response. Molecular mechanisms involve glutathione depletion and reactive oxygen species (ROS) production as key factors leading to pulp or gingival cell apoptosis.

Moharamzadeh et al (2009)[28] reviewed the biocompatibility of restorative dental materials and their components, and a wide range of conventional as well as new technique test systems for the evaluation of the biological effects of these materials. Oral and mucosal adverse reactions to resin-based dental materials have been reviewed.

Polydorou et al (2009)[34] investigated the elution of BisGMA, TEGDMA, UDMA and BPA from nanohybrid, ormocer and a chemically cured composite material at different storage periods and found that there is a decrease in the elution of TEGDMA after 28 days and 1yr whereas BISGMA release was same even after 1yr. Monomer elution from ormocer is less compared to the other materials.

Miletic Vesna et al (2009)[26] correlated the monomer elution and ratio of carbon-carbon double bonds from monomer to polymer (RDB) from different adhesive systems. Monomer elution was quantified using reverse phase high performance liquid chromatography and RDB obtained using Raman Specctroscopy. 90% of monomer elution occurred during first 24 hours. RDB was significantly less immediately after curing when compared with 24 h and 7 days. In all the adhesive systems RDB increased after monomer elution. It was concluded that there is no direct relation between RDB and monomer elution in adhesive systems.

Ahmed et al (2010)[1] evaluated the percentage of apoptotic cells in the epithelium of buccal and labial mucosa after applying amalgam and composite filling materials. The epithelial cells were

stained with fluorescence dyes; ethidium bromide, propidium iodide and monoclonal antiFas-1 antibody then examined under fluorescent microscope. The cytotoxicity of amalgam was decreased with aging time while that of composite was increased. On the other hand, using antifas-1 antibody, it was found that the apoptotic cells were died through mitochondrial pathway.

Manojlovic et al (2011)[22] studied the monomer elution from microhybrid, nanohybrid and ormocer based composites cured with halogen light, LED light sources for varied time intervals from 1 h to 28 days by using high performance liquid chromatography. It was found that more amount of monomer elution occurred from nanohybrid composites compared to the ormocer and microhybrid composites. The light sources showed no variation in monomer elution except for nonohybrid composite Tetric EvoCeram which showed more elution of monomers when cured with LED light source.

Djuricic et al (2011)[8] studied the relation between the degree of conversion (DC) and the elution of substances from three different resin based cements using Raman Spectroscopy and High Performance Liquid Chromatography (HPLC). There is no significant

difference in the degree of conversion between the three resin based cements. But significant difference was noted in the amount of monomer elution between the resin cements. It was concluded that no relation exists between the degree of conversion and amount of eluted substances.

Schneider et al (2011)[38] investigated the degradation resistance of silorane, pure ormocer and dimethacrylate based resin composites. Water sorption, solubility and color stability parameters were also compared between the composites. It was concluded that the color stability of silorane and ormocer composites was inferior to that of dimethacrylate based composites. But the silorane exhibited lower water sorption and solubility compared to ormocer and dimethacrylate based composites.

Deb Sanjukta et al (2011)[7] evaluated if pre-warming of composites can influence the flow, marginal adaptation and other properties of the material. The flow and the degree of conversion of the composites were enhanced after pre-warming but the flow extent varied among the materials. The polymerization shrinkage increased while no changes are seen in flexural strength. Better marginal

adaptation of the composites was seen due to increased flow but the incidence of microleakage was unaltered.

Van Landuyt et al (2011)[46] reviewed the literature on the short- and long-term release of components from resin-based dental materials, and to determine how much (order of magnitude) of those components may leach out in the oral cavity. While the release of monomers was analyzed in many studies, that of additives, such as initiators, inhibitors and stabilizers, was seldom investigated. Significantly more components were found to be released in organic than in water-based media. Resin-based dental materials might account for the total burden of orally ingested bisphenol A, but they may release even higher amounts of monomers, such as HEMA, TEGDMA, BisGMA and UDMA. Compared to these monomers, similar or even higher amounts of additives may elute, even though composites generally only contain very small amounts of additives. A positive correlation was found between the total quantity of released elutes and the volume of extraction solution.

Durner et al (2011)[9] tested the hypothesis that realistic concentration of bisphenol-A-glycidyldimethacrylate (BisGMA), triethyleneglycol dimethacrylate (TEGDMA), 2-hydroxyethyl

methacrylate (HEMA) and methyl methacrylate (MMA) found in elution experiments can cause DNA strand breaks in human gingival fibroblasts (HGP). Such DNA damage was compared with that resulting from ionizing radiation coming from natural sources, dental radiography or tumor therapy. TEGDMA, HEMA and MMA did not induce DNA strand breaks at concentrations of up to 10 mM. About 24 h after incubation with 0.25 mM BisGMA, significantly more DNA strand breaks were found in HGP compared to controls.

Hegde et al (2012)[17] evaluated the release of BisGMA and TEGDMA from two flowable composite materials (Esthet X-Flow and Tetric N-Flow) under different polymerization time periods of about 20s, 30s, 40 s for storage periods of about 24 hours and 7 days. There was no significant difference in elution of monomers with regard to different polymerization time periods of 20s, 30s, 40s. Elution of TEGDMA from Tetric N-Flow and Esthet X-Flow was more in 24 hours than 7 days. But elution of BisGMA from Esthet X-Flow was more in 24 hours than 7 days whereas in Tertic N-Flow higher amount of BisGMA eluted from 7 days compared to 24 hours.

Michelsen et al (2012)[25] quantified the monomers released in saliva after restoration with composite material at the interval of 10

mins, 24 hours and 7 days. Monomers BisGMA, UDMA, HEMA and TEGDMA were detected in samples of saliva collected after 10 mins. But no monomers were detected in samples collected after 24 hours and 7 days.

Rahim et al (2012)[35] evaluated the effect of acidic drinks (orange juice and coke) on the diffusion coefficient, water sorption and solubility characteristics of various composite materials (Filtek Z250, Spectrum TPH 3 and Durafill VS). Most composites showed significant increase in water sorption after immersion in coke and orange juice. When immersed in coke, Spectrum TPH 3 showed increase in solubility while Durafill VS showed the highest solubility.

Materials and Methods

MATERIALS

1. Filtek™ Z100 – shade A3 (3M ESPE, USA)
2. Filtek™ Z250 XT - shade A3 (3M ESPE, USA)
3. Filtek™ Z350 XT – shade A3 (3M ESPE, USA)
4. Admira – shade A3 (VOCO, Germany)
5. HEMA – 2 -Hydroxy ethyl methacrylate (cas no. 128635 , Sigma-Aldrich co., UK)
6. TEGDMA – Triethylene glycol dimethacrylate (cas no. 261548 , Sigma-Aldrich co., UK)
7. UDMA – Diurethanedimethacrylate (cas no. 436909 , Sigma-Aldrich co., UK)
8. BisGMA – Bisphenol A glycerolatedimethcrylate (cas no. 494356 , Sigma-Aldrich co., UK)
9. 75% Ethanol.
10. 0.01M Potassium dihydrogen phosphate in water
11. Acetonitrile
12. Dulbecco's Modified Eagle Medium. (Hi-Media ™:AT068)
13. Fetal Bovine Serum (Invitrogen™)
14. Antibiotics and Antifungal agents

Penicillin – 100 IU/ml

Streptomycin - 100µg/ml

Amphotericin B - 100µg/ml

15. D -PBS –Dulbecco's Phosphate Buffered Saline (potassium chloride-0.2g/l, potassium phosphate monobasic – 0.2g/l, sodium chloride – 8g/l , sodium phosphate dibasic – 1.15g/l)
16. Trypsin 1:125 (Tissue culture grade , Hi media ^{TM})
17. Ethylene Diamine Tetra Acetic Acid (Hi media ^{TM})
18. MTT - 3-(4, 5-dmethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide.
19. DMSO – Dimethyl sulphoxide

ARMAMENTARIUM:

1. Teflon mould
2. Glass plate
3. Matrix strips
4. Electronic balance (Dhona 200 D^{TM})
5. Glass vials
6. Volumetric flask
7. Pipette

8. BP blade no 15
9. Centrifuge
10. Suction pump
11. 0.22 µm pore size filter paper (Sartorius stedim)
12. -20° deep freezer (cryoscientific)
13. Autoclave (LabMartin)
14. Hot air oven
15. Culture dishes
16. 96 well plate
17. Incubator
18. Digital camera (Sony Cybershot , 7.1 MP , 3X Zoom)

SPECIAL EQUIPMENTS:

1. Halogen curing unit (Elipar ™ 2500 , 3M ESPE)
2. Ultrafast liquid chromatography (Prominence – XR, Shimadzu)
3. Phase contrast microscope (Olympus CKX41 ™, USA)
4. Carbon di-oxide incubator (Thermo electron corporation, Forma Series II water jacketed – HEPA class 100, USA).
5. Laminar flow cabinet (Clean Air)
6. Plate reader (BIO – RAD model 680)

METHODOLOGY

Sample preparation:

Four different composite restorative materials – microhybrid (Filtek Z100, 3M ESPE), ormocer (Admira, VOCO), nanohybrid (Filtek Z250 XT, 3M ESPE) and nanocomposite (Filtek Z350, 3M ESPE) were investigated. The composition of these materials and their manufacturers are listed below.

MATERIAL	MANUFACTURER	TYPE	COMPOSITION
Filtek Z100 ™ Shade A3	3M ESPE (St. Paul MN, USA)	Microhybrid	Mixture of BisGMA, TEGDMA and inorganic fillers
Admira shade A3	Voco GmbH (Cuxhaven , Germany)	Ormocer	Mixture of UDMA, BisGMA , ormocers and silicate fillers.
Filtek™ Z250 XT shade A3	3M ESPE (St. Paul MN , USA)	Nanohybrid	Mixture of BisGMA , UDMA , TEGDMA , BIS-EMA and inorganic fillers.
Filtek™ Z350 XT shade A3	3M ESPE (St. Paul MN , USA)	Nanocomposite	Mixture of BisGMA , UDMA , TEGDMA , BIS-EMA and nanoscale fillers.

Cylindrical moulds made of teflon of diameter 5mm and height 2mm were used. Eight samples for each of the four composite materials were prepared. The Teflon moulds were placed on the matrix strips over the glass plate. The composite materials were then added to the teflon moulds in one increment. Then the matrix strip was placed, over which the glass plate was placed to get a flat surface. The matrix strip was placed to prevent the formation of oxygen – inhibiting layer. The materials in the teflon moulds were then cured with halogen light (Elipar^{TM} 2500, 3M ESPE) for 40 seconds according to the manufacturers instructions. After curing the samples were weighed using electronic balance (Dhona 200 D^{TM}). Then the samples were immersed in glass vials containing 2 ml of 75% ethanol and incubated at 37°C for 24 hours. After 24 hours the samples were removed and the solution was sent for analysis by high performance liquid chromatography (HPLC).

High Performance Liquid Chromatography (HPLC) analysis:

High performance liquid chromatography instrument (Ultrafast Liquid Chromatography, Prominence-XR, Shimadzu) which is equipped with column Enable C-18 (150 x 4.6mm, 5µm particle size)

was used for the qualitative and quantitative analysis of the solution. The mobile phase was a mixture of 0.01M Potassium dihydrogen phosphate in water and acetonitrile. Ethanol was used as the diluent. The flow rate was 800 µl /min with the injection volume about 10 µl. The monomers were identified by comparing their retention times with retention times of the reference compounds. But this should be done under same HPLC conditions. The standard compounds of HEMA, TEGDMA, UDMA and BisGMA were obtained and standard stock solutions were prepared.

Monomer standard stock preparation

Standard solution A

Weighed accurately 8 mg/ml of 2-Hydroxyethyl methacrylate and transferred into a 5.0 ml volumetric flask, dissolved and made up to 5.0ml using ethanol.

Standard solution B

Weighed accurately 7 mg/ml of Triethylene glycol dimethacrylate and transferred into a 5.0 ml volumetric flask, dissolved and made up to 5.0 ml using ethanol.

Standard solution C

Weighed accurately 20 mg/ml of Diurethane dimethacrylate and transferred into a 5.0 ml volumetric flask, dissolved and made up to 5.0ml using ethanol.

Standard solution D

Weighed accurately 10 mg/ml of Bisphenol A glycerolate dimethacrylate and transferred into a 5.0 ml volumetric flask, dissolved and made up to 5.0ml using ethanol.

Preparation of Standard mixture

Standard solution E

Accurately pipette out 200 µl of standard solution A , 250µl of Standard solution B, 100 µl of Standard solution C , 200µl of Standard solution D and made up to 1.0 ml with ethanol.

Standard solution F

Accurately pipette out 500 µl of solution E and made up to 1.0 ml with ethanol. Standard solution F is used as monomer standard for the analysis. Now the samples were passed and the results were

evaluated according to the peak areas. The results were recorded in ppm.

The obtained data were tabulated and statistically analyzed using One - way analysis of variance (ANOVA) and post hoc tukey test with a significance level of $P<0.05$.

Isolation and culture of human gingival fibroblast

Healthy human gingival tissue was obtained from patient undergoing crown lengthening procedure following informed consent from the patient. Under local anesthesia, a small portion (2 x 1 x 1 mm) of gingiva was removed using a scalpel. The tissue was placed in a nutritional medium (Dulbecco's modified eagle medium, DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml and amphotericin B 100 µg/ml) and taken to the cell culture laboratory. The tissue was then rinsed in sterile phosphate buffer saline (PBS, pH = 7.4) and transferred to a petridish containing DMEM. The tissue was minced mechanically using a scalpel. The obtained suspension of tissue was condensed by centrifugation (2,500 rpm for 5 min). The pellet obtained was placed in a culture dish in culture medium (DMEM)

containing 10% FBS with antibiotics and incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

Trypsinization

After obtaining confluency, media was removed from the plate and the cells were washed with PBS. 3 ml of Trpsin/EDTA solution was added and kept at 37°C for 3 minutes. The whole content was transferred to a centrifuge tube and centrifuged at 2,500 rpm for 5 min. To the pellet 1ml of DMEM media with 10% FBS was added. The cell numbers were determined and their viability was assessed by the tryphan blue dye exclusion test.

Monomer solution preparation

Four dental composite resin monomers were used in this study. They were Bisphenol A glycerolate dimethacrylate, Triethylene glycol dimethacrylate, 2-Hydroxyethyl methacrylate and Diurethane dimethacrylate. All the four monomers were dissolved in DMSO and diluted with culture medium based on the different concentrations required by serial dilution. The maximum concentration of DMSO used was 0.5%.

Cytotoxicity by MTT assay

MTT assay was performed to determine the mitochondrial dehydrogenase activity. Cells were seeded into a 96 well plate in 200µl of DMEM media at 5 x 10^3 cells for 24 h with 10% FBS. Different concentrations of individual monomers were then treated with the cells for 24 h at 37˚C in a humidified atmosphere of 5% CO2 in air. DMSO treated cells and untreated cells served as controls. After incubation, 100 µl of culture medium was removed and 25 µl of MTT stock solution (5 mg/ml in PBS) was added to each well. The plates were incubated for 4 hours at 37˚C and 5% CO_2. All medium was removed from each well and 200 µl of lysis buffer was added to each well. The plates were covered in aluminium foil and placed at 100˚C for 20 min. After cooling, the plates were read at 570 nm on a plate reader. The experiment was repeated a minimum of five times. . Results were calculated as 100 (X/control), where X is the average reading of a single treatment group. Then the mean value and standard deviation was calculated. They were schematically represented using bar diagrams with the concentration along the X-axis and percentage of viable cells along Y-axis. IC50 concentration was determined from the bar diagram which is the drug concentration that is required to reduce the viability to half that of the control.

METHODOLOGY FLOWCHART

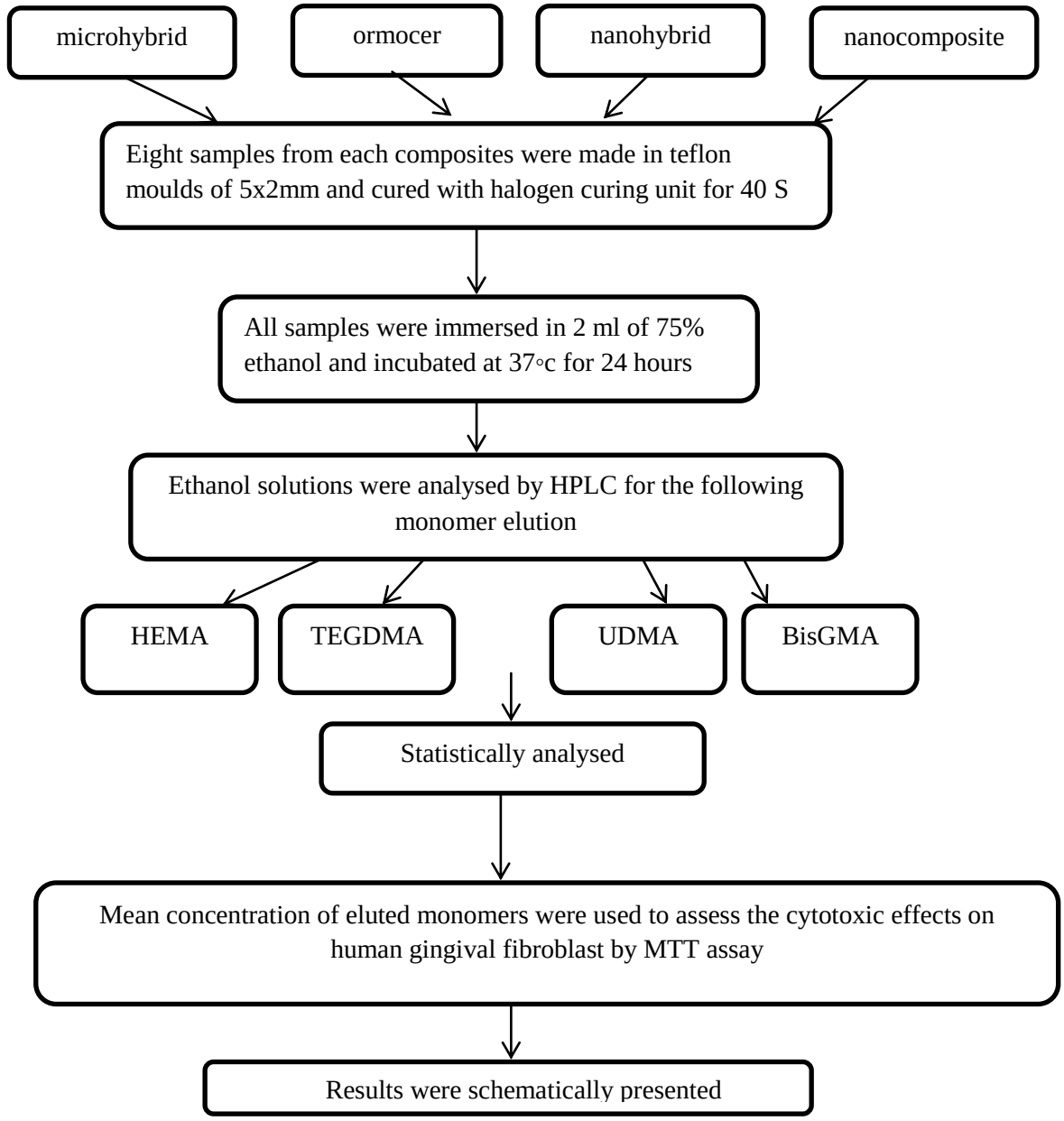


Fig 1: Composite resins and Teflon mould

Fig 2: Standard Monomers

Fig 3: 75 % Ethanol

Fig 4: DMSO and MTT

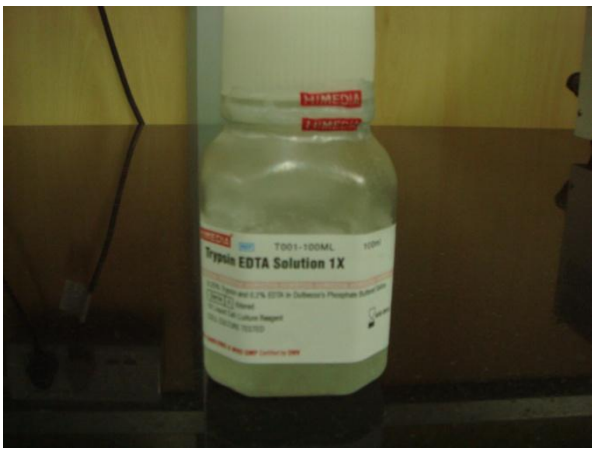


Fig 4: Trypsin and EDTA solution

Fig 5: 0.22µm pore filter paper

Fig 7: Dulbecco's Modified Eagle Medium

Fig 8: Fetal Bovine Serum and culture medium

Fig 9: Halogen light curing unit

Fig 10: Electronic Balance

Fig 11: Incubator

Fig 12: HPLC unit

Fig 13: Centrifuge

Fig 14: Phase Contrast Microscope

Fig 15: Carbon di-oxide Incubator

Fig 16: laminar Flow Cabinet

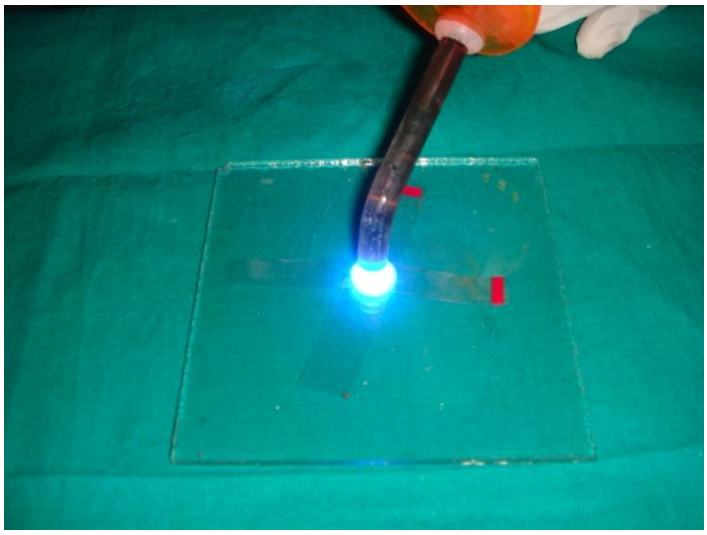


Fig 17: Curing the composite resin in teflon mould

Fig 18: Samples for HPLC analysis

Fig 19: Site of Tissue Collection

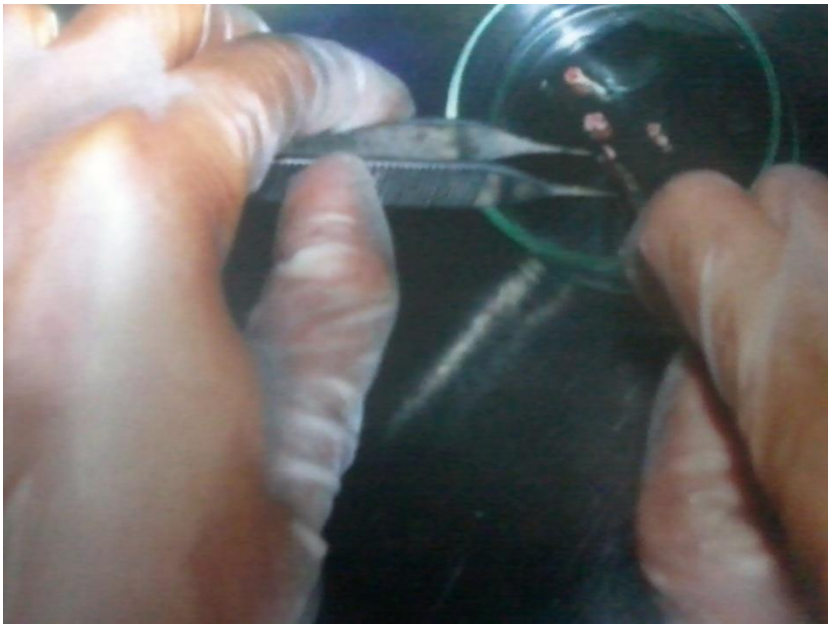


Fig 20: Tissue minced by scalpel

Fig 21 : Incubation of culture dish

Fig 22: Cell counting using contrast phase microscope

Fig 23: Incubation of the 96 well plate covered with aluminium foil

Fig 24: Plate Reading

Figures

Table 1: Comparison between monomers eluted within each composite for 24 hours

Composites	Concentration of monomers in ppm				P value
	HEMA	TEGDMA	UDMA	Bis GMA	
Microhybrid	77.50±9.02[a]	1634.13±97.60[c]	29.63±6.25[a]	729.38±98.19[b]	<0.001**
Ormocer	54.00±8.77[a]	472.50±39.98[b]	1180.13±59.18[c]	1515.25±82.33[c]	<0.001**
Nanaohybrid	55.00±6.12[a]	108.75±14.26[a]	1122.00±64.97[b]	1210.63±70.51[b]	<0.001**
Nanocomposite	74.50±7.43[a]	422.25±34.51[a]	2540.88±143.08[b]	2417.00±222.46[b]	<0.001**

NOTE:

1) ** denotes significance at 1% level

2) Different alphabets denote significance at 5% level using tukey HSD test.

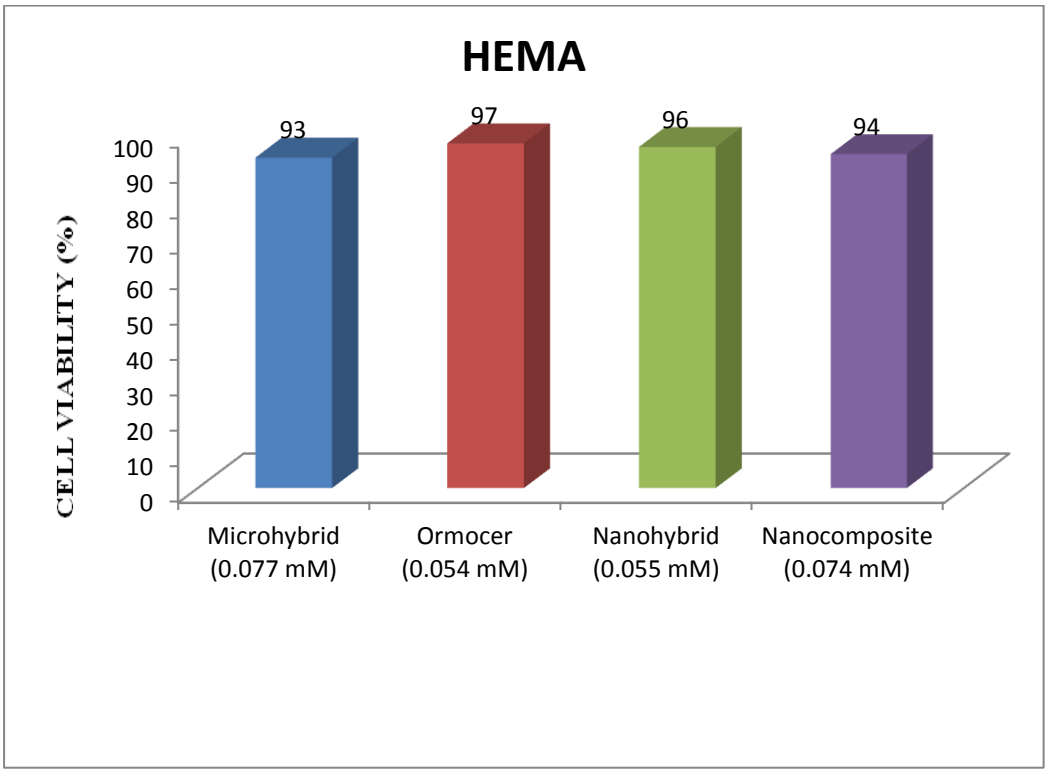
Table 2 : Comparison of each monomer eluted between the composites for 24 hours.

Composites	Concentration of monomers in ppm			
	HEMA	TEGDMA	UDMA	Bis GMA
Microhybrid	77.50 ± 9.02^{b}	1634.13 ± 97.60^{b}	29.63 ± 6.25^{a}	729.38 ± 98.19^{a}
Ormocer	54.00 ± 8.77^{a}	472.50 ± 39.98^{a}	1180.13 ± 59.18^{b}	1515.25 ± 82.33^{b}
Nanaohybrid	55.00 ± 6.12^{a}	108.75 ± 14.26^{a}	1122.00 ± 64.97^{b}	1210.63 ± 70.51^{ab}
Nanocomposite	74.50 ± 7.43^{b}	422.25 ± 34.51^{a}	2540.88 ± 143.08^{c}	2417.00 ± 222.46^{c}
P value	0.032*	<0.001**	<0.001**	<0.001**

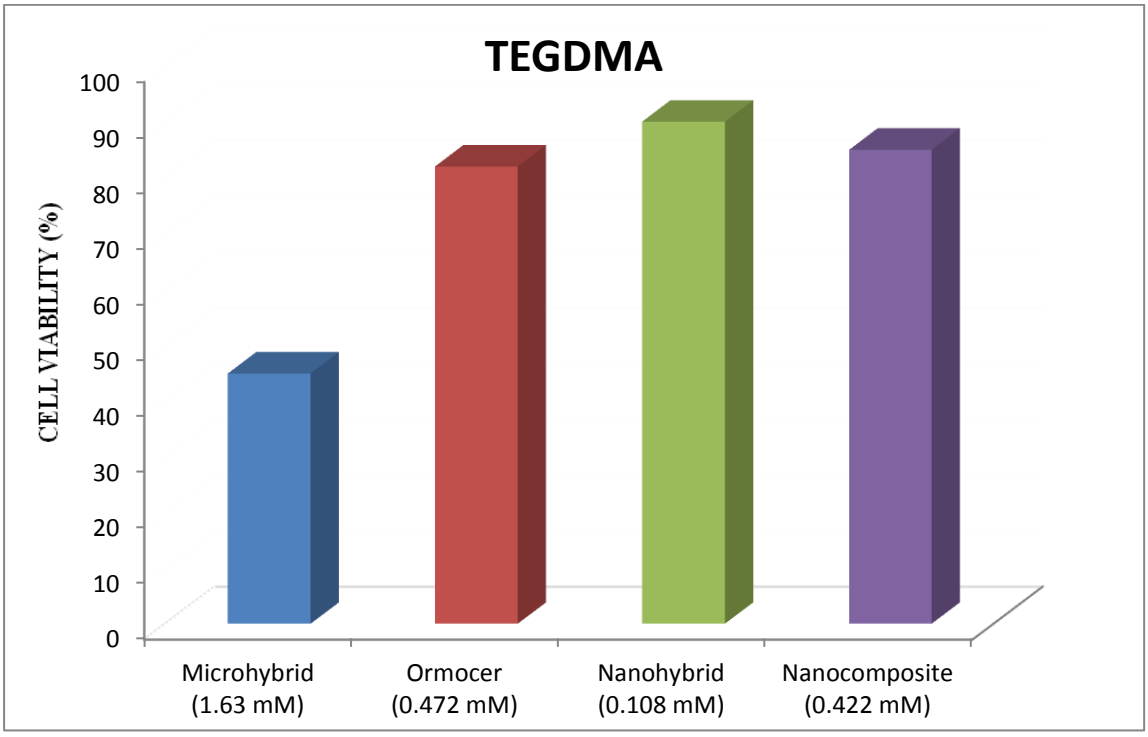
NOTE:

1) ** denotes significance at 1% level
2) * denotes significance at 5% level
3) Different alphabets between days denote significance at 5% level using tukey HSD test.

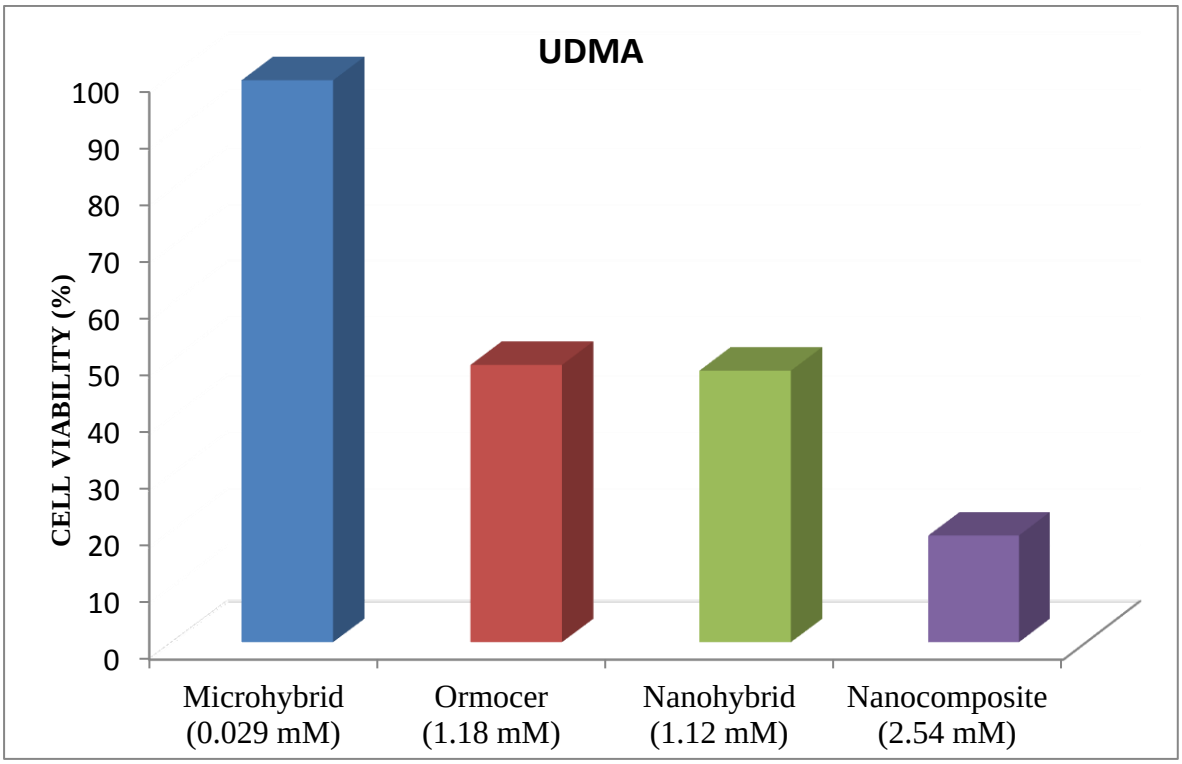
GRAPH 1: PERCENTAGE OF VIABLE CELLS AFTER EXPOSURE TO HEMA



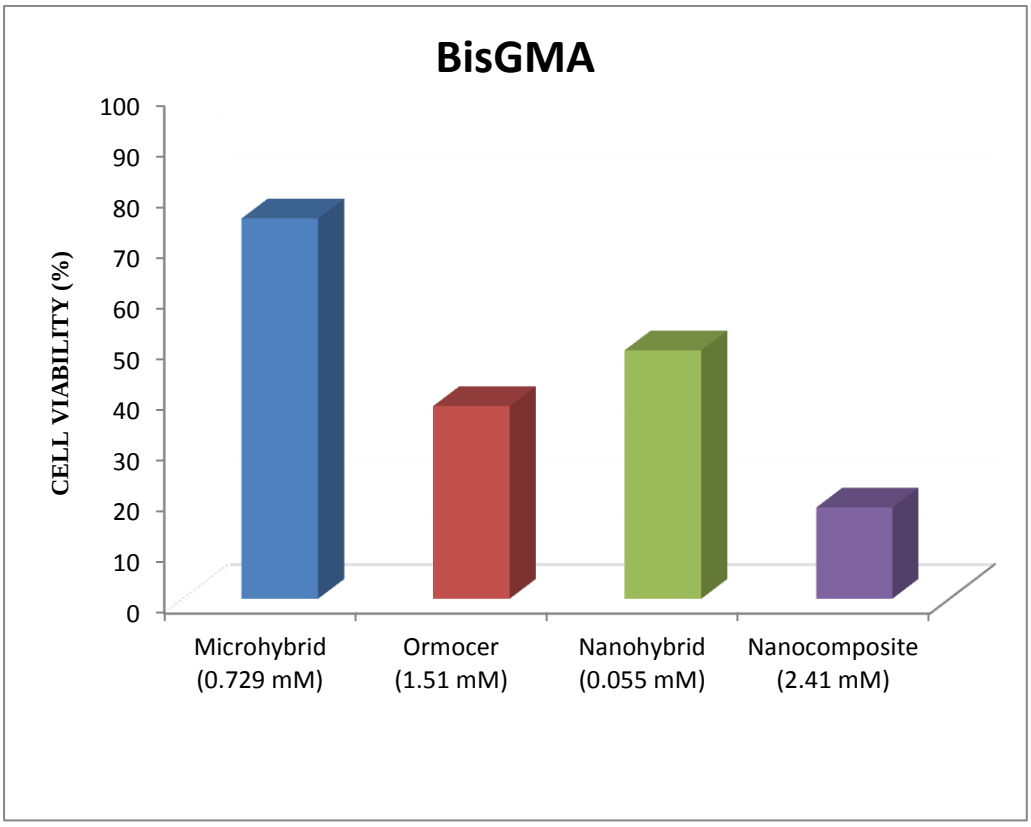
GRAPH 2: PERCENTAGE OF VIABLE CELLS AFTER EXPOSURE TO TEGDMA



GRAPH 3: PERCENTAGE OF VIABLE CELLS AFTER EXPOSURE TO UDMA



GRAPH 4: PERCENTAGE OF VIABLE CELLS AFTER EXPOSURE TO BISGMA



Results

RESULTS

For comparing the monomer elution within each composite and between the composites the obtained values were statistically analysed using One - Way ANOVA and Post Hoc Tukey HSD tests. The ANOVA technique is used to compare the numerical means of two or more samples. It tests the null hypothesis that samples in two or more groups are drawn from populations with same mean values. The One-Way ANOVA in particular is used to test a minimum of three groups. Tukey HSD test is a single step multiple comparison procedure used in conjunction with ANOVA to find means that are significantly different from each other.

Table 1 shows the comparison between monomers eluted within each composite. In microhybrid (Filtek Z100, 3M ESPE) composite, more amount of TEGDMA was eluted followed by BisGMA. HEMA and UDMA were eluted in very least quantity. There was statistically significant difference between the amount of eluted TEGDMA and BisGMA (significant at $P<0.001$). In ormocer (Admira, Voco) composite, more amounts of BisGMA and UDMA were eluted followed by TEGDMA. HEMA was eluted in least

quantity. There was no statistically significant difference in the elution of BisGMA and UDMA ($P>0.05$). In nanohybrid (Filtek Z250, 3M ESPE) composite, similar amounts of BisGMA and UDMA were eluted. Least quantities of TEGDMA and HEMA were eluted. In nanocomposites (Filtek Z350, 3M ESPE) more amounts of UDMA and BisGMA are eluted with no statistically significant difference between them ($P>0.5$).

Table 2 shows the comparison of each monomer eluted between the composites. When the elution of monomer HEMA is compared between the composites there is no statistically significant difference in the concentrations eluted between the four different composites ($P<0.05$). In comparing the TEGDMA elution between the composites, there is more amount of TEGDMA eluted from microhybrid compared to other composites (statistically significant at $P<0.001$). Greater amount of UDMA is eluted from the nanocomposites followed by the ormocer and nanohybrid. There is no significant difference in the amount of UDMA eluted between the ormocer and nanohybrid composites. When the elution of BisGMA is compared, nanocomposites elute higher amount followed by ormocer, nanohybrid and microhybrid composites. There is statistically

significant difference in the amount of BisGMA elution between the composites (P<001).

The mean concentrations of the monomers evaluated by HPLC was used to assess the cytotoxicity of the monomers on human gingival fibroblasts by MTT assay. The results were schematically represented by bar diagrams.

Graph 1 shows the percentage of viable cells when exposed to the HEMA concentrations eluted from four different composites. All the four concentrations showed least cytotoxicity as the percentage of viable cells were more than 90%.

Graph 2 shows the percentage of viable cells when exposed to TEGDMA concentrations from the four different composites. The concentration of TEGDMA from microhybrid composite seems to be more cytotoxic as its cell viability was 45%. The concentrations from the other three composites were comparatively less cytotoxic.

The percentage viability of cells exposed to UDMA concentrations from the four composites are depicted in **Graph 3** The concentration of UDMA eluted from microhybrid composite showed no cytotoxic reactions. The other three concentrations showed to be

more cytotoxic as their cell viability was less than 50%. In this , UDMA eluted from nanocomposite was highly cytotoxic which showed cell viability of 19%.

The percentage of viable cells exposed to BisGMA concentrations eluted from four different composites are depicted in **Graph 4**. BisGMA eluted from microhybrid composite was comparatively less cytotoxic than the other composites. Concentration eluted from nanocomposite was highly cytotoxic as it showed only 18% cell viability.

Discussion

DISCUSSION

The introduction of adhesive technology and tooth colored restorative materials has reduced the popularity of amalgam, in day to day practice. The versatile nature of the tooth colored restorative materials has made its application more frequent and wider.

Composite restorative materials in addition to providing esthetic solution also promised to achieve functional requirements of strength, volumetric and morphologic stability, physical compatibility with the surrounding tooth structure, biocompatibility and the ability to self adhere to the tooth surface. The dentists prefer tooth colored restorative material because it favours minimal tooth preparation which conserves the tooth structure.[23]

Since the evolution of resin based dental composites 50 years ago, its composition has evolved significantly. Earlier the changes were mainly done in the filler content. The filler size is reduced to produce materials with effective polishing property and to increase the wear resistance. Later changes were made on the polymeric matrix of the material to develop composite systems with reduced polymerization shrinkage stress.[12]

Dental composites are differentiated by their requirements as restoratives, sealants, cements, provisional materials, etc. These materials are similar in their basic composition which includes a polymeric matrix, a dimethacrylate, reinforcing fillers, silane coupling agent and few additives to control polymerization reaction.[34]

The organic part or the polymerizable resin matrix consists of one or more base monomers like BisGMA or UDMA with diluents co-monomers like TEGDMA, EGDMA, HEGDMA.[33] The monomer system is the main backbone of the composite resin system[15]. There are differences in the composition of monomers used for each composite material and this difference affects the reactivity, viscosity, polymerization shrinkage and the mechanical properties of the composite material.[34]

Each type of composite material are differentiated by the variations in size of reinforcing fillers. According to the filler size the composites are classified as "macrofill" composites, "microfill" composites, "microhybrid" composites and "nanohybrid" composites. Recently "nanofill" composites are developed which contain only nanoscale particles.[12]

A high percentage of filler content improves the physical and mechanical properties of the organic matrix. The various other functions of the filler content are that, it reduces the thermal coefficient of expansion and overall curing shrinkage, provides radio-opacity, improves the handling and esthetic properties[15]. The commonly used fillers include silica, glass, quartz or ceramic material.[37]

The composite restorative material in addition to the resin matrix, fillers and organosilane also contains various additives. Photoinitiators and co-initiators (e.g Dimethyl – Aminobenzoic acid-Ester) are present to initiate the polymerization reaction. Camphoroquinone is the most commonly used photoinitiator system. But recently some commercial materials are using other photoinitiator systems such as PPD (1-phenyl-1,2-propanedione), Lucirin TPO (monoacylphosphine oxide) and Irgacure 819 (bisacylphosphine oxide) which are more color stable as they are less yellow than Camphoroquinone. Inhibitor system like Hydroquinone Monomethyl Ether are present to maximize the storage period of composite prior to curing and photostabilizers like Benzophenone to provide color stability by eliminating the effect of UV light.[12]

Recently a new resin system called Ormocer has been introduced. Ormocers are ORganically MOdified CERamics which is a hybrid structure made by combining organic and inorganic components at nanoscopic level by sol-gel method. This combination of organic and inorganic components enhances their resistance against chemical degradation. Dimethacrylates are also added to the ormocers.[3,38]

In dental composites, the viscous resin is converted to a rigid set material by free radical polymerization of the methacrylate monomers either by thermal, chemical or photochemical methods.[37] Photopolymerization of the resins is the most common method. In photopolymerization method, the composite material set via an addition polymerization reaction (i.e) the material on exposure to light of particular wavelength and intensity, initiates the generation of free radicals which propagates the polymerization leading to a set material. The degree of polymerization by photoinitiation method depends on the wavelength and intensity of light output, curing time, the size, location and orientation of tip of light source, shade, thickness and composition of material.[6]

The various light sources available in the market today are Halogen lamps, Plasma arc lamps, LED lights and the Laser. Halogen and LED lamps are the most commonly used sources for photopolymerization.[31] The Halogen lamp was used in this study as it is a known fact that dental composites cured with halogen light require minimum energy density to allow optimum curing.[6] The degree of polymerization of halogen light is higher than the LED curing unit.[2] Less than optimum properties have been obtained when plasma arc lamp is used.[6,29,30]

The amount of monomer getting converted to polymer during polymerization is termed as the "Degree of Conversion". The degree of conversion is never a complete process. Literatures give evidence that there is only about 40% to 70% conversion of monomer to polymer occur during polymerization.[22] The factors which influence the degree of conversion can be divided as material related factors and clinician related factors. The material related factors are composition of monomers, concentration of activator and inhibitor present, viscosity of monomers, diffusion limitation of reactive media present and size, shade, opacity of filler particles. The clinician related factors are light intensity of the curing unit, duration of light

irradiation, temperature produced during polymerization and thickness of restorative material[12,17,30]

The majority of unreacted monomers in the cured material is attached chemically to the network in the form of pendant side groups.[4] About 10% of the monomers are trapped within the polymers as unreacted monomers and oligomers.[8,22] These unreacted monomers elute from the resin based composites as a result of chemical biodegradation. According to Ferracane, the process of elution depends on three main factors namely the extent of polymerization reaction (i.e) degree of double bond conversion, chemistry of the solvent, the size and chemical nature of the eluted components.[12,42]

For the evaluation of monomer elution, various solvents such as distilled water, saliva, ethanol, methanol and acetonitrile has been used in earlier studies.[10,17,22] In this study ethanol was used as a solvent as 75% ethanol is approved by US Food and Drug Administration as a saliva substitute and commonly used organic elution medium. Also ethanol has the solubility parameter which matches that of BisGMA. A match in solubility parameter results in maximum softening of the resin. Ethanol penetrates the polymer

network, causes expansion of the structure during the release of uncured monomers.[12,23,22,34] The solvent disrupts the secondary inter chain bonds but not the primary cross link bonds. Storing composites for 24 hours in ethanol did not affect the mechanical properties.[47]

Eluted monomers from composite resin are of high clinical significance because of their cytotoxic effects to gingival fibrobalsts and macrophages.[18,25,27] Studies cited in the literature has confirmed, the release of unpolymerized monomers eluted from polymerized composite resin as a source of wide variety of adverse biological reactions including local and systemic toxicity , pulp reactions , allergy and oestrogenic effects[16]. Stomatitis, swelling of lips and perioral dermatitis had been cited.[25] There are three ways of systemic intake of eluted substances from the resin based composites: (1) Ingestion through the gastro - intestinal tract, (2) Diffusion to the pulp via dentinal tubules and (3) Inhalation of volatile components in the lungs.[46] The cytotoxic effect has been ranked on the base of monomers in the following manner: BisGMA > UDMA > TEGDMA > HEMA.[5,18]

The aim of this present study was to evaluate the amount of release of four monomers – BisGMA, UDMA, TEGDMA and

HEMA from four different composite restorative materials using High Performance Liquid Chromotography and to assess the cytotoxicity of these monomers on human gingival fibroblasts by MTT assay.

The four different composite material examined in this study are microhybrid, nanohybrid, ormocer and nanocomposites. These four composite materials were chosen based on their evolution. Various studies have been done on the monomer elution from microhybrid composites but scarce data are available on the monomer elution from nanohybrid and ormocer based composites.[22] No literature is available on the monomer elution from newly introduced nanocomposites (Filtek Z350 XT).

The sample preparation for placing resin composites are in accordance with previous studies using teflon mould of height 2mm and diameter 5mm.[24,30] To prevent the formation of oxygen inhibited superficial layer, a transparent plastic matrix strip was placed over the material filled mould during curing.[25,31] The materials are cured with halogen lamp for 40s as instructed by the manufacturer. Then the samples were weighed and immersed in glass bottles containing 2 ml of 75% ethanol. The reason for choosing ethanol as the solvent

was described earlier. Glass bottles were used to avoid false positive results which may occur if plastic containers were used as it is a polymer based material.[46]

The samples were then incubated at 37°c for 24 hours. 24 hours had been taken as the elution time because most of the studies has reported that nearly all the leachable components were eluted within first 24 hours from the composites.[46] Ferracane and Condon has reported about 85% - 100% of monomer elution within first 24 hours.[10] Yap et al has shown that hydrolysis process is the reason for any elution to take place after 24 hours of curing.[48]

For qualification and quantification of eluted monomers from the four composite material High Performance Liquid Chromatography has been used in this study. Because of its wide availability and less economic fact, High performance Liquid Chromatography is the most commonly used method than Gas Chromatography with Mass Spectrometry. Literature states that Gas Chrmatography with Mass Spectrometry is more suited for the detection of low molecular weight elutes whereas High Performance Liquid Chromatography is helpful in the detection of high molecular

weight elutes.[8,24,26,32] Also the detection limit and specificity of High Performance Liquid Chromatography is higher than that of Gas Chromatography with Mass Spectrometry.[46]

The result of this study by HPLC analysis shows that higher amount of BisGMA was eluted from all the four composites. komurcuoglu et al and Polydorou et al also reported more amount of BisGMA elution compared to other monomers in their study[20,33] The more elution of BisGMA was explained by the fact that the double bond conversion of BisGMA is lower compared to other monomers (Stansbury et al).[45] Also the elution medium used was 75% ethanol which has the solubility parameter similar to that of BisGMA.[33]

The elution of UDMA was similar to that of BisGMA in nanohybrid (Filtek Z250 XT) and Ormocer (Admira) composites. This was in par with the findings reported by Manojlovic et al.[22]

The elution of TEGDMA was more in microhybrid (Filtek Z100) composite compared to the other three composites. In microhybrid composite the amount of TEGDMA eluted was more than the BisGMA. This was similar to the result reported by Darmani et al.[5] But Munksgaard et al reported similar amount of TEGDMA

and BisGMA elution from microhybrid (Filtek Z100) composite.[30] The elution of TEGDMA was less in other three composites. This was explained by the fact that TEGDMA is a diluent monomer which is generally added to decrease the viscosity of the composite.[35]In microhybrid composites the filler size are larger which results in high viscosity. Hence more amount of plasticizers like TEGDMA are added to decrease the viscosity. But in the nanohybrid and nanocomposites the filler particles are in the nanoscale levels which does not increase the viscosity and hence lower quantity of TEGDMA are added in these composites which results in lower elution.[24]

In all the four different composites least quantity of HEMA was eluted. Though it is not present in the manufacturers composition data, it was eluting in minor quantities from all the composites. This is in agreement with the results obtained by Michelsen et al and Manojlovic et al who reported the elution of HEMA.[22,24] Spahl et al and Michelsen et al explained that least amount of HEMA was eluted as a degradation product of UDMA even though it is not an ingredient of a resin based composite.[24,44]

When the monomer elution from all the composites were compared, nanocomposites (Filtek Z350 XT) showed higher elution than the other three composites. No literature data is available with regard to monomer elution from the newly introduced Filtek Z350 XT. But the high amount of monomer elution may be due to the fact that the volume of filler content is less and resin content is more in Filtek Z350 XT compared to the other three composites.

To assess the cytotoxicity of resin based dental materials, cell culture studies were used. Various assays are available to measure the viability and proliferation status of cells in relation to material cytotoxicity. MTT assay is the most widely used test because of its simple, rapid and inexpensive nature. MTT assay shows cell viability by alterations of mitochondrial enzyme, succinate dehydrogenase activities. It converts water soluble methylthiazole tetrazolium bromide to an insoluble purple formazan.[28] In this study MTT assay was chosen to assess the cytotoxicity of the four monomers – HEMA, TEGDMA, UDMA, BisGMA on human gingival fibroblasts which is in line with the study done by Issa et al and Darmani et al.[5,18]

The cytotoxicity of the monomers were tested on human gingival fibroblasts because they are in close proximity with

composite restorations especially in class II and class V cases. Also biological assessment of dental materials are frequently done on fibroblasts (Theilig et al, Polyzois et al).[27] The exposture time of human gingival fibroblasts culture to the monomers also plays an important role in assessing the cytotoxicity.[36] In the present study, the gingival fibroblast culture was exposed to the monomers for 24 hours as the monomer elution had been assessed for 24 hours which was considered to be the time interval where most of the monomer elution occurs as said by previous Ferracane et al.[11] Hence the maximum toxic effects will be seen in this period.

In the previous studies (Moharamzadeh et al, Issa et al) done to assess the cytotoxicity on human gingival fibroblasts, arbitrary concentrations of the different monomers were used and the IC 50 values were calculated.[11,18] But in the present study the mean concentrations of the four monomers – HEMA, TEGDMA, UDMA, BisGMA obtained from four different composites after 24 hours by HPLC analysis was used.

The result shows that BisGMA was the most cytotoxic monomer when compared to other three monomers. The concentrations of BisGMA obtained from the four different

composites are high and hence has resulted in more cytotoxicity. The cytotoxic nature of BisGMA is mainly due to its increased liposoluble nature. In general the monomers exhibit its cytotoxicity by affecting the permeability of cell membranes mainly by altering the lipid layers of the cell membrane (Schuster et al).[39] The nanocomposite eluted the maximum concentration of BisGMA and hence found to be more cytotoxic whereas the concentration eluted by microhybrid composite was low and thus less cytotoxic as confirmed by this present study.

The monomer UDMA which had eluted in similar concentrations to BisGMA in Ormocer, nanohybrid and nanocomposite is the next cytotoxic monomer. In nanocomposite though the concentration of UDMA eluted is slightly more than BisGMA, its cytotoxicity is comparatively less than BisGMA because UDMA is less liposoluble than BisGMA (Schuster et al).[39] The concentration of UDMA eluted from microhybrid composite is very less and hence it exhibited no cytotoxicity in this assay.

Various studies has projected TEGDMA as one of the most cytotoxic monomer. Moharamzadeh et al has found TEGDMA to be more cytotoxic than UDMA even though TEGDMA is less

liposoluble than UDMA.[27] Also TEGDMA induces apoptosis (Spagnulo et al)[43] and interferes with glutathione activity (Lefeuvre et al).[21] In the present study, only microhybrid composite eluted more concentration of TEGDMA and hence exhibited more cytotoxicity. The other three composites eluted less TEGDMA and hence found to be less cytotoxic.

All the four composite materials used in this study eluted least concentrations of HEMA and thus the cytotoxicity elicited by this monomer was very low. The ranking for the cytotoxicities of the monomers were found to be BisGMA > UDMA > TEGDMA > HEMA which was in agreement with the previous studies (Issa et al , Darmani et al).[5,18] In this study the cytotoxicity of the monomers were mainly influenced by the concentrations of these monomers released from the four different composites.

Summary

SUMMARY

This study was done to evaluate and quantify the elution of four different monomers – HEMA, TEGDMA, UDMA, BisGMA from four different composites after 24 hours by high performance liquid chromatography. The cytotoxicity of these eluted monomers on human gingival fibroblasts was assessed by MTT assay. The four composites examined were microhybrid (Filtek Z100), Ormocer (Admira), nanohybrid (Filtek Z250 XT) and nanocomposites (Filtek Z350 XT). Eight samples from each composite were made in Teflon mould of size 5 mm in diameter, 2 mm in thickness and cured with halogen light for 40 s. All the samples were immersed in glass vials containing 2 ml of 75% ethanol and incubated at 37°C for 24 hours. At the end of 24 hours the samples were removed and the solution was given for high performance liquid chromatography analysis. The results were tabulated and statistically analysed using one – way ANOVA and Post hoc Tukey HSD test. The mean concentrations of each monomer for the four composites were obtained.

Healthy human gingival tissue was obtained following informed consent from the patient undergoing crown lengthening

procedure. Fibroblast culture was done using this tissue. The cultured cells were then transferred to 96 well plate and was exposed to the concentrations of the four monomers – HEMA, TEGDMA, UDMA, BisGMA for 24 hours. DMSO treated cells and untreated cells served as controls. The plates were then read on a plate reader at 570 nm. The experiment was repeated for five times. The mean value of percentage of viable cells for the eluted monomer was calculated. The results were then schematically represented using bar diagrams with the percentage of viability cells along the Y- axis and the concentrations of the each monomer from four composites on the X- axis.

Conclusion

CONCLUSION

Within the limitations of this study, it was concluded that

1. Different composites elute different quantity of monomers.
2. The newly introduced nanocomposite Filtek Z350 XT (3M ESPE) eluted the maximum amount of monomers at the end of 24 hours compared to the other three composites.
3. BisGMA was the monomer that was eluted in high concentrations from ormocer, nanohybris and nanocomposite.
4. TEGDMA was found to elute in high concentration from microhybrid composite.
5. BisGMA was observed to be the most cytotoxic monomer in this study.
6. The ranking for the cytotoxicity of the four monomers can be given as BisGMA > UDMA > TEGDMA > HEMA.

Most of the studies has used arbitrary concentrations of the monomers to assess its cytotoxicity. But the uniqueness of this study was that the quantity of monomer eluted at the end of 24 hours was recorded by HPLC analysis and the same quantity was used to assess its cytotoxic effect on human gingival fibroblasts using MTT assay.

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